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TITLE: The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

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Breast cancer develops in two stages. The first, hyperproliferation, causes primary tumors and is rarely lethal. In the second stage, malignancy, tumor cells penetrate the surrounding basement membrane layer of extracellular matrix proteins and migrate into adjacent tissues where they form secondary, metastatic tumors. The defining characteristics of malignant cancer cells are hyperproliferation and an aggressively *migratory* phenotype.
We have two models whereby an immortal, non-metastatic breast epithelial cell can be induced to constitutively migrate on its preferred adhesive substrate, laminin-5. The first model is by direct activation of the laminin-5 integrin receptor $\alpha 3\beta 1$ with a $\beta 1$-integrin activating antibody. The second is by modification of laminin-5 by specific proteolytic cleavage, an event known to occur in cancerous tissues. This project's goal is to analyze the contribution of the signaling molecules FAK and CAS to the cascade that induces migration of breast cells on laminin-5. In the first phase of this research we determined that FAK and CAS are phosphorylated upon binding of breast cells to laminin-5, and that phosphorylation is enhanced when these cells are bound to the cleaved form of laminin-5. The focus of the current work is to analyze these molecules in the model of direct integrin activation.

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## Table of Contents

**The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5**

## I. Introduction

The defining characteristic of malignant cancer cells are hyperproliferation and an aggressively migratory phenotype. We have two models whereby an immortal, non-metastatic breast epithelial cell can be induced to constitutively migrate on its preferred adhesive substrate, the basement membrane matrix molecule laminin-5. The first model is by direct activation of the laminin-5 integrin receptor $\alpha 3\beta 1$ with a $\beta 1$-integrin activating antibody. The second is by modification of laminin-5 by specific proteolytic cleavage, an event known to occur in cancerous tissues. We are utilizing these two model systems to analyze alterations in integrin-linked signaling events that occur when a cell is stimulated to migrate.

## II. Body

### Statement of Work

Task 1. Analyze FAK activation and CAS phosphorylation following adhesion of MCF-10A's to pro-migratory, cleaved vs. uncleaved laminin-5 matrix.

a. Phosphorylation/activation state of endogenous FAK will be determined by immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies and in vitro kinase assay following a time-course after adhesion to laminin-5.

***Task 1 Progress Report: Preliminary experiments showed enhanced phosphorylation of both FAK and CAS following adhesion of MCF-10A's to cleaved, pro-migratory laminin-5. These experiments were carried out using immunoprecipitation with specific FAK or CAS antibodies followed by Western blotting with anti-phosphotyrosine antibodies. In vitro kinase assays to measure activation of FAK have not been completed to date.***

Task 2. Analyze FAK activation and CAS phosphorylation following stimulation of MCF-10A's with $\beta 1$-integrin activating antibody TS2/16.

**Task 2 Progress Report: In preliminary experiments, I was unable to repeat the previously reported TS2/16-stimulated migration of MCF-10A's on laminin-5. To test if this was due to the batch of antibody that I was using I prepared a fresh stock of the antibody from serum-free cell culture supernatants using a Protein G-Sepharose chromatography. The new stock of antibody has allowed me to repeat the experiments showing enhanced migration of 10A's on laminin-5. Analysis of FAK and CAS phosphorylation have not been completed.**

Task 3. Determine role of exogenously expressed FAK, CAS and respective mutants on migration of MCF-10A's.

a. Stable cell lines will be generated by transfection of MCF-10A's with expression vectors encoding epitope-tagged versions of FAK, CAS and variants.

**Task 3a Progress Report: Three attempts were made in the past year to obtain stable MCF-10A cell lines over-expressing FAK, CAS and variants. FAK and CAS cDNA's were subcloned into pcDNA3.1 (Invitrogen), a eukaryotic expression vector containing a CMV-promotor and G418 drug resistance marker. In all attempts I was able to obtain numerous G418 resistant clones, but no clones over-expressed either FAK or CAS. The expression vectors were determined to be functional by transient transfection of COS cells. I am currently pursuing**

**transient transfection experiments of MCF-10A cells using these vectors in combination with GFP as a marker for transfection efficiency.**

b. Analyze migration of cell lines on laminin-5 and other extracellular matrix molecules.
c. Corroborate data generated from stable cell lines with analysis of same constructs in transient transfection assays.

Task 4. Determine role of migration-linked signaling pathways in laminin-5 cleavage model.
a. Analyze cAMP levels in MCF-10's plated on cleaved vs. uncleaved laminin-5
b. Analyze activation of ERKs during migration on cleaved laminin-5

**Task 4 Progress Report: Task 4 experiments have not been attempted to date.**

Additions to original Statement of Work:

A) While performing experiments associated with Task 1 it became evident that much variation existed in stocks of cleaved laminin-5 that were produced by enzymatic digestion of purified matrix. To address this issue I am in the process of constructing a recombinant laminin-5 γ2 chain that is truncated at precisely the point of enzymatic cleavage. This construct contains a 6-His tag at the carboxyl terminus and a signal peptide at the amino terminus. A full-length γ2 chain construct with a 6-His tag is also being constructed. The goal is to express either the full-length or truncated molecule in either MCF-10A or the rat bladder carcinoma cell line 804G, which both express the laminin-5 β3 and α3 chains. Laminin-5 will be purified from cell culture supernatants using metal chelate affinity chromatography. Using this system I should be able to obtain laminin-5 preparations that only contain the cleaved or the uncleaved γ2 chain. Potential problems with this system are: 1) Improper or non-functional association of exogenously expressed truncated γ2 chain with endogenous α and β chains. However, evidence from the literature suggests that the exogenously expressed γ2 chain should associate normally with the endogenous α and β laminin chains. 2) Inability to purify sufficient quantities of recombinant laminin-5 using this system.

B) Laminin-5 is not commercially available at this time. We have relied on an industry resource to obtain this material for our experiments. So I would not be dependent on this source alone, I have set up a system to purify laminin-5 from 804G cell culture supernatants using an antibody affinity column. (laminin-5 γ2 chain specific monoclonal antibody TR1). I have been able to isolate pure, soluble laminin-5 using this system and will use this as a back up if needed.

**III. Key Accomplishments** July 1, 2000 – June 30, 2001:

- Analysis of FAK and CAS phosphorylation following adhesion of MCF-10A's to promigratory, cleaved vs. uncleaved laminin-5.
- Determination that MCF-10A cells will not tolerate exogenously expressed FAK, CAS and variant molecules.
- Construction of a recombinant cleaved laminin-5 γ2 chain cDNA.

**Reportable Outcomes** July 1, 2000 – June 30, 2001.

Publications and Abstracts:

Earley, B., Plopper, G.E. and J.L. Huff. The role of FAK in breast cell migration on laminin-5. Western Alliance of Medical Students Annual Meeting 2001.

Plopper, G.E., J.L. Huff, W.L. Rust, M.A. Schwartz and V. Quaranta. 2001. Antibody induced activation of β1 integrin receptors stimulates cAMP-dependent migration of breast cells on laminin-5. *Molecular and Cellular Biology Research Communications* **4**, 129-135.

Student Training:

Brian Earley, undergraduate Biology major, did an independent research project under my supervision from May 2000 to March 2001. He submitted an abstract on his project and presented a talk at the Western Alliance Medical Student Conference held in February in Carmel, CA. In addition, Brian was awarded an undergraduate research grant for $1000.00 for supplies from UNLV for work done on this project. Brian is currently applying for admission to medical school.

Kilpatrick Carroll, undergraduate Chemistry major, did independent research under my supervision during the first year of this grant. He worked extensively on the attempts to make MCF-10A cell lines. He is enrolled in graduate school at Columbia University for Fall 2001.

Michael Cascia, undergraduate Biology major, assisted in this project for a 3 month period (March 2001-May 2001). Michael utilized the laboratory research skills that he acquired to obtain employment as a laboratory technician at UCSF.

**IV. Conclusions**:

Cell migration, though it is widely recognized as an integral part of tumor cell metastasis, is a largely untapped area for chemotherapy drug design. Our research is focused on identifying cellular signaling events that specifically control migration of normal and malignant breast cells. We expect that by delineating signaling events controlling migration on a biologically relevant matrix molecule, that we may gain useful information for the development of anti-migratory drugs in the future.